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The activation of the periplasmic (NiFe) hydrogenase of *Desulfovibrio gigas* by carbon monoxide

Yves M. Berlier*, Guy D. Fauque*, Jean LeGall*+, Paul A. Lespinat* and Harry D. Peck, Jr+

*Section Enzymologie et Biochimie Bactérienne, ARBS, CEN Cadarache, 13108 Saint Paul lez Durance Cedex, France and +School of Chemical Sciences, Department of Biochemistry, University of Georgia, Athens, GA 30602, USA

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The activation of the periplasmic (NiFe) hydrogenase from *Desulfovibrio gigas* by dihydrogen is a complex phenomenon involving both 'slow' and 'fast' reactions. Carbon monoxide, a competitive inhibitor of hydrogenase activity, is demonstrated to cause the slow activation nearly as well as dihydrogen. Carbon monoxide does not reduce the (NiFe) hydrogenase and the fast reductive activation is effected by deuterium in the exchange assay. In the presence of dithionite, which immediately reduces the redox centers of the (NiFe) hydrogenase, the slow activation is still essential to attain full activity. Thus, the slow non-reductive and fast reductive steps of the activation can occur in any sequence.

Hydrogenase; Proton-deuterium exchange; Enzyme activation; Carbon monoxide; (*Desulfovibrio gigas*)

1. INTRODUCTION

Previous studies [1–4] reported that the (NiFe) periplasmic hydrogenase from *Desulfovibrio gigas* becomes inactivated during isolation and storage and must undergo a reactivation process requiring H_2 in order to regain full activity in H_2-uptake and -production assays or in the H^+-D_2 exchange reaction. Two inactivation-activation processes with different time courses have been postulated [3], involving three states of the enzyme, 'unready', 'ready' and 'active'. The unready state [5] or 'form 1' [6] has been correlated with the presence of nickel EPR signal A and proposed to represent either the 'oxidized enzyme in the wrong conformation' [7] or an oxygenated form of the oxidized hydrogenase [6]. The ready state [5] or 'form 2' [6] has been correlated with nickel signal B and suggested to represent either the hydrogenase in the right conformation for activity but with the hydrogen-activating site in too high an oxidation state [8] or a deoxygenated form of the oxidized hydrogenase [6]. Both ideas are based on the slow conversion, requiring several hours, of the unready state (form 1) to the ready state (form 2) (primary activation) in the presence of hydrogen or mild reducing agents and the rapid conversion of the ready state (form 2) to the active state of the hydrogenase under reducing conditions (secondary activation).

This work provides evidence that the slower primary activation process can be achieved, in the absence of hydrogen, by CO although the latter gas is an inhibitor of hydrogenase activity [9] and is unable to ensure the reduction of the hydrogenase. This result indicates that the full activation of this hydrogenase can clearly be separated into two processes: a non-reductive primary process possibly involving the replacement of a gaseous inhibitory ligand such as oxygen and a secondary reductive process catalyzed by hydrogen or strong reducing agents. Dithionite, which mediates the instantaneous reduction of the redox centers of the (NiFe) hydrogenase, does not induce full activity and the slow primary activation effected by CO or H_2 is still required for maximum

Correspondence address: Y.M. Berlier, ARBS, CEN Cadarache, F-13108 Saint Paul lez Durance Cedex, France

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activity of the enzyme. Thus, the primary non-reductive activation and the secondary reductive activation are demonstrated to be independent phenomena.

2. MATERIALS AND METHODS

Experiments were performed with the periplasmic hydrogenase from *D. gigas* (NCIB 9332) [10].

Hydrogenase activity was measured in the proton-deuterium exchange assay using a reaction vessel connected to the ion source of a VG model 8-80 mass spectrometer via a membrane inlet allowing in-line measurements of the concentrations of dissolved gases [11]. A nitrogen/deuterium mixture (80:20, v/v) prepared from commercial nitrogen (L'Air Liquide, grade N30) and from 98.5% deuterium (Oris, Saclay) and freed from oxygen traces by passing through a photoreduced liquid trap [12] was used to equilibrate the buffer in the reaction vessel (0.1 M phosphate, pH 7.6, 30°C).

An adequate enzyme dilution was flushed by the activating gas, H_2, Ar or CO (same origin and grade) and aliquot samples equivalent to 2–10 μg hydrogenase were removed at intervals and injected into the D_2-equilibrated buffer. The exchange reaction resulted in the disappearance of D_2 (mass peak 4) and appearance of HD and H_2 (mass peaks 3 and 2) which were followed using a peak-jumping device monitored by an Apple IIc microcomputer. The exchange rates were calculated from the initial slopes of the corresponding curves and the results, expressed as μmol HD plus H_2 evolved per min, were referred to the protein content of the enzyme preparation [13].

3. RESULTS AND DISCUSSION

A possible role of CO in hydrogenase activation was suspected when that gas, known as an inhibitor of hydrogenase activity [9], was found not to inhibit but rather to enhance the reductive activation by dihydrogen of the periplasmic hydrogenase from *D. gigas* (fig.1) and to exert a protective effect against a decrease of that activity with time during storage (not shown). CO alone was able to activate *D. gigas* hydrogenase as revealed by subsequent H^+-D_2 exchange assays

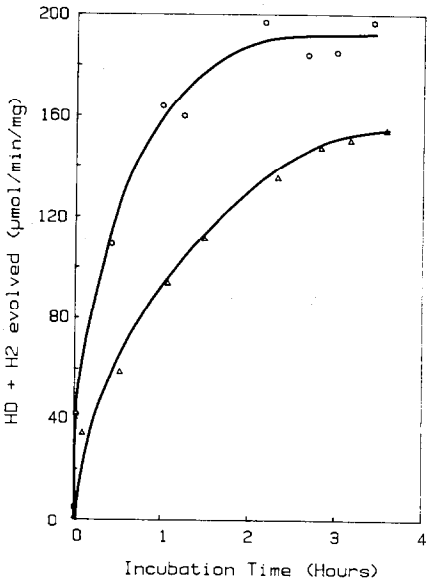

Fig.1. CO effect upon the reductive activation by H_2 of the (NiFe) periplasmic hydrogenase of *D. gigas*. A hydrogenase sample was incubated under an atmosphere of: curve 1 (○), 20% H_2 in CO; curve 2 (△), 20% H_2 in Ar. The aliquot samples used in the H^+-D_2 exchange assay were 1.84 μg. See the text for the conditions.

(fig.2, curve 2). The activation pattern was not very different from that observed in the presence of H_2 (fig.2, curve 1) although no concomitant reduction of the enzyme, as observed during H_2 activation [1], was caused by CO. An opposite pattern was obtained with dithionite which instantaneously reduced but only partly activated the enzyme while full activation required almost as much time as with H_2 or CO (fig.2, curve 3).

The activation pattern of hydrogenase can therefore be viewed as follows: the slow process is non-reductive but is involved with the binding of a gas, possibly replacing oxygen, to the enzyme active sites; the fast process is reductive and can be accomplished by different reductants but it is independent of the non-reductive step. Among the different activating agents, dihydrogen alone is able to carry out both processes simultaneously [1].

CO is also a biologically active molecule and several sulfate reducers contain a CO dehydrogenase which in all anaerobic bacteria tested is a (NiFe-S) protein [14]. The low redox potential of the CO_2/CO couple (−560 mV) could

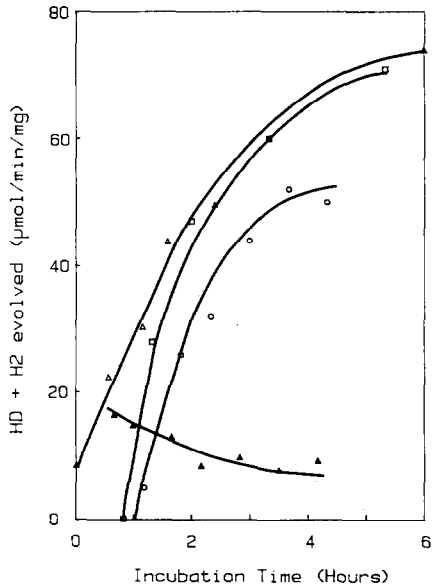

Fig.2. Compared activation of the (NiFe) periplasmic hydrogenase of *D. gigas* with: curve 1 (□), pure H_2; curve 2 (○), pure CO; curve 3 (△), 0.5 mM $Na_2S_2O_4$; curve 4 (▲), pure Ar (in the latter case, the H^+-D_2 exchange assay was performed with an extemporaneous addition of 0.5 mM $Na_2S_2O_4$ in the reaction vessel). See the text and fig.1 for the conditions.

be compatible with a reductive activation of hydrogenase by CO in a manner similar to hydrogen. However, no spectrophotometric evidence for the reduction of (Fe-S) centers was observed during the course of hydrogenase activation under CO. On the other hand, CO, which is a competitive inhibitor of hydrogenase activity, probably also competes with O_2 for the active centers. A similar idea has been advanced to explain the protective effect of CO against oxygen inactivation observed with a partially purified hydrogenase [15]. Argon, an inert gas with regard to metals, is ineffective in the activation of hydrogenase (fig.2, curve 4) except for a mechanical scrubbing of oxygen which may shorten or even suppress the lag phase in the H_2-mediated activation [1]. It should be noted that the argon used in these studies contained larger traces of hydrogen than the CO, thus eliminating contaminating hydrogen in the CO as a source of error. The rapid reductive activation of the hydrogenase from the ready state to the active state is presumed to occur when the hydrogenase is brought into contact with the deuterium-nitrogen gas mixture used for the exchange assay. The amount of CO transferred from the activation reaction mixture to the exchange reaction vessel resulted in a concentration of CO which was of the order of micromolar and had no inhibitory effect on the (NiFe) hydrogenase [1].

The demonstration that the slow and fast activators are independent processes has important implications for the mechanisms involved in the activation of the (NiFe) hydrogenase. An interaction between CO and the oxidized forms of the (NiFe) hydrogenase has not been demonstrated by spectroscopic techniques; however, the slow activation effected by CO suggests that such an interaction must occur. CO has been shown to modify nickel signal C ($g = 2.19$) of the partially reduced hydrogenase [16]. This effect of CO has been extensively studied with the (NiFe) hydrogenase of *Chromatium vinosum* and shown to be light-sensitive [17]. With the *D. gigas* enzyme, in the presence of dithionite, the EPR signals originating from the nickel and three-iron cluster disappear and EPR signals of the four-iron centers are observed [6]. The fact that the slow activation is observed in the presence of dithionite as well as in its absence indicates that it is independent of the oxidation states of the four-metal redox centers of the (NiFe) hydrogenase. These observations tend to support the idea that a conformational change is responsible for the slow primary activation of the (NiFe) hydrogenase; however, one must still assume some type of binding of CO and H_2 as effector of the change. Because the species responsible for the nickel A and nickel B signals disappear upon reduction with dithionite but the slow activation still occurs, it would appear that these paramagnetic species may not reflect different activated forms of the (NiFe) hydrogenase.

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